ADVANCED HEALTHCARE MATERIALS

Supporting Information

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Rapid Self-Integrating, Injectable Hydrogel for Tissue Complex Regeneration

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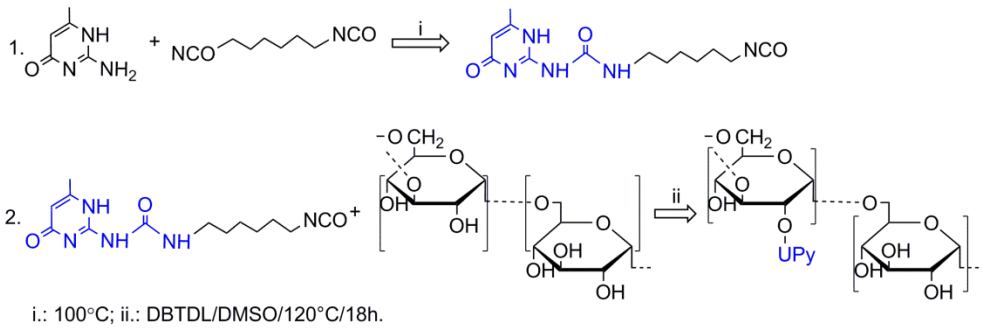

Scheme 1. Synthesis steps of DEX-UPy polymers.

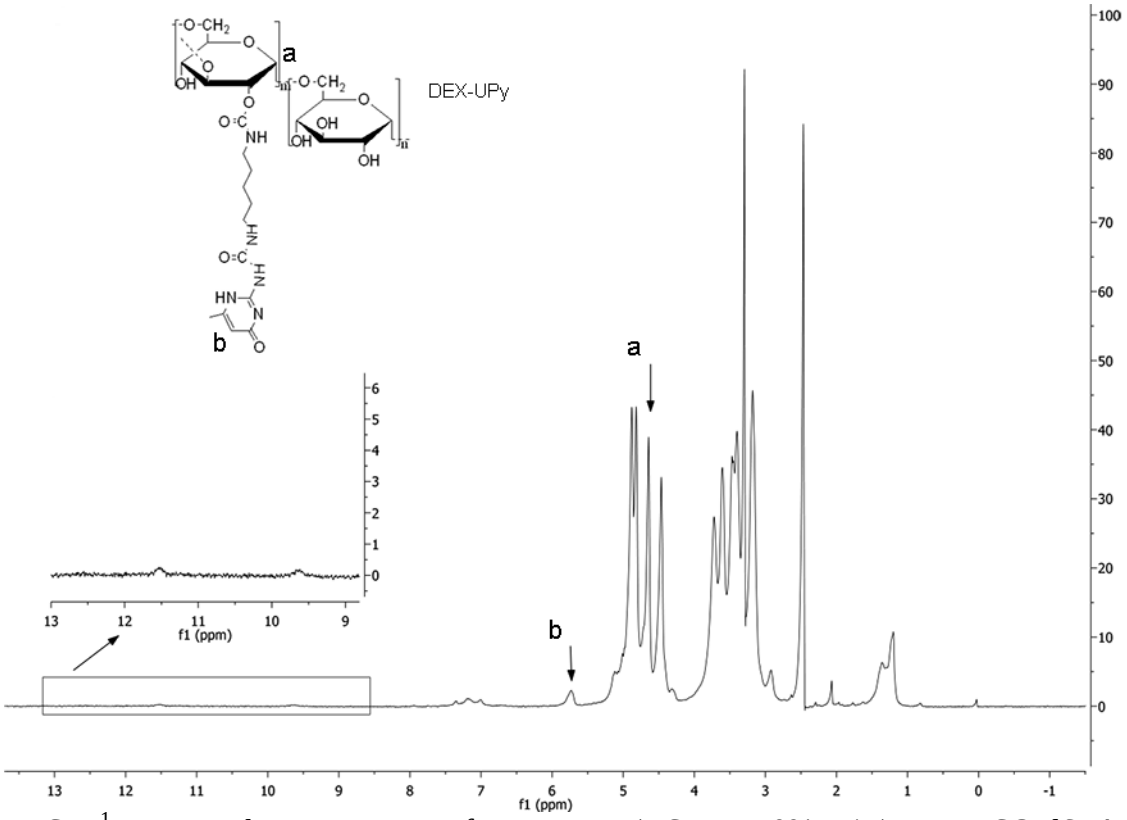

Figure S1. ^{1}H NMR characterization of DEX-UPy (DS 5.5, 10% w/w) in DMSO-d6. A peak appears at around 11.5 ppm (in the magnified inset), which is a hydrogen-bonding signal, demonstrating the formation of UPy dinners.

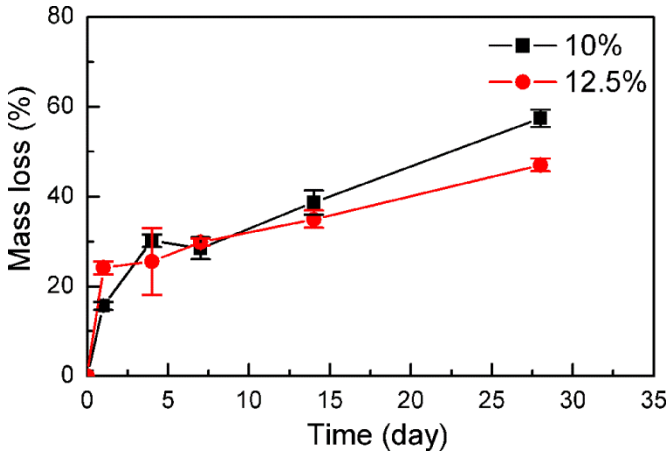

Figure S2. In-vitro degradation of DEX-UPy hydrogel in PBS at 37℃. Hydrogels (DS 5.5) with concentrations of 10% w/w (black square) and 12.5% w/w (red circle).

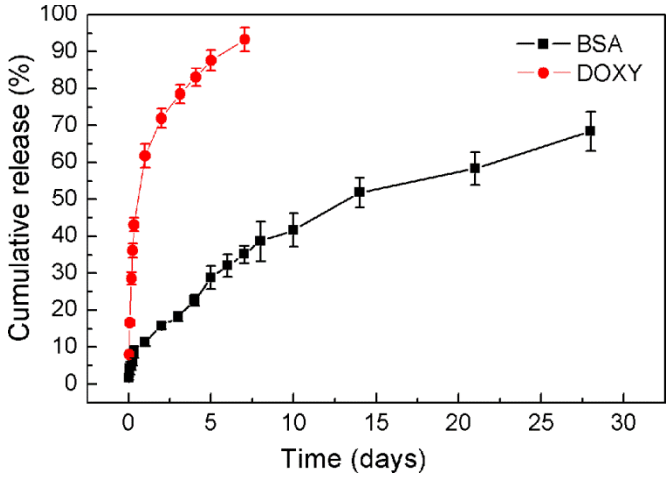

Figure S3. In-vitro release of BSA and DOXY from the DEX-UPy polymer (DS 5.5, 10% w/w) hydrogel in PBS at 37℃. BSA (black square), DOXY(Red circle).

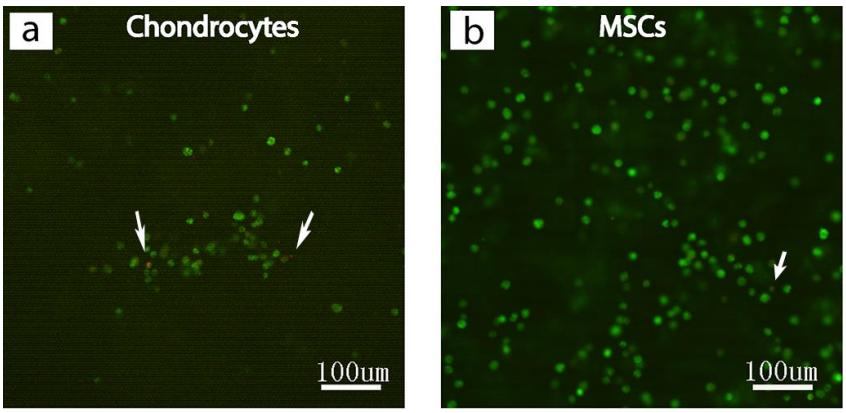

Figure S4. Live-dead assay for cells encapsulated in DEX-UPy hydrogels (DS 5.5, 10% w/w) after in culture for 2 weeks. (a) Chondrocytes, (b) BMSCs. Green dots in the image represent live cells while red dots represent dead cells.

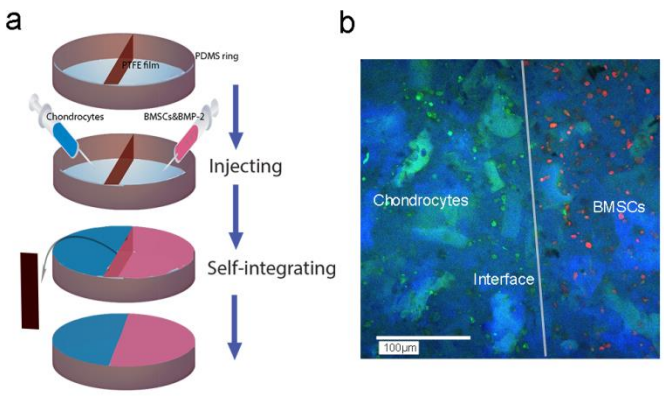

Figure S5 The preparation of a self-integrated hydrogel construct encapsulating chondrocytes and BMSCs. (a) Schematic illustration of the process of the self-integration of the two portions after being injected into the mold. (b) A confocal image of the cells encapsulated in the hydrogel construct. Chondrocytes were stained green and BMSCs were stained red. Two parts of the hydrogel were well integrated with interface in the middle.

Materials and Methods

Materials. All the chemicals were purchased from Sigma-Aldrich (USA) unless specified otherwise. Dialysis tubes (MWCO6000-8000) were purchased from Fisher Scientific (USA). Bone morphogenetic protein-2 (BMP-2) was purchased from Peprotech (Rocky Hill, New Jersey).

Synthesis of DEX-UPy polymers. Ureido-pyrimidinone (UPy) bearing an isocyanate group was synthesized using a published method, through the reaction of 2-amino-4-hydroxy-6-methylpyrimidine with 1,6-hexanediisocyanate.[1] Then UPy was grafted to the dextran (DEX) backbone through the reaction of the isocyanate group with a hydroxyl group on the DEX. To synthesize the UPy grafted DEX, typically the DEX (2 g, MW70,000) was dissolved in 70 ml anhydrous dimethyl sulfoxide (DMSO) under nitrogen atmosphere and magnetic stirring,

followed by the addition of UPy-isocyanate (0.4 g, Sigma) and dibutyltin dilaurate (0.586 ml). The reaction was carried out at 120℃ for 18h. The resulting solution was poured into isopropanol (700 ml) to precipitate for 3 times. The powder was centrifuged and re-dissolved in water, followed by dialyzing against deionized water for 3 days. Light yellow powder was obtained after lyophilization for 3 days and stored in vacuum for later use.

NMR characterization. 1H NMR characterization was carried out using the Varian MR400 (Cobalt) Spectrometer in the Department of Chemistry at the University of Michigan. CDCl3 was used as the solvent for UPy-isocyanate and DMSO-d6 for DEX-UPy.

Preparation of hydrogel. To fabricate hydrogels, typically 100 mg polymer was dissolved in 900 µl PBS (pH7.4) at 70℃ for 1 hour under magnetic stirring. Afterwards, the solution was loaded into a syringe and injected into a PDMS mold. For demonstration and rheological property measurement, the hydrogel was put in a refrigerator at 4℃ overnight to allow for complete gelation. For the demonstration of self-healing, one of the hydrogel disks was dyed with rodamine (red) to better visualize the interface. Hydrogel disks were cut with a blade and rejoined together manually. Photos were taken 2 minutes after the rejoining.

Rheological test. The rheological properties of the hydrogels were measured using an AR2000 rheometer (TA instruments, United States). Parallel plate with 40 mm diameter was used for all the tests. The gap distance between the plates was 0.4 mm. A constant stress of 0.1 Pa was applied for the frequency spectrum measurement. For measurements other than frequency spectrum, a constant 1 rad/s angular speed was used. The high stress and low stress used in the

shear-thinning and self-recovery experiments were 100 Pa and 0.1 Pa respectively. Self-recovery of the modulus was validated after 3 cycles of high and low stress. For the temperature stability test, the modulus during the heating process was measured with a heating rate of 2℃/min from room temperature to 70℃.

***In vitro* erosion.** After the hydrogels were loaded into syringes and stored in a refrigerator at 4℃ overnight, they were injected into 1.5ml eppendorf tubes. For every 100 mg hydrogel in a tube, 1 ml PBS (pH7.4) was added. The tubes were incubated on a shaker with a shaking speed of 100 rpm in an incubator at 37℃. At each predetermined time point, 3 samples were collected and freeze-dried. The dry weights were measured on a balance, accurate to 0.1 mg. The dry weight loss was calculated to quantify the erosion.

***In vitro* drug release.** The DEX-UPy powder was dissolved into PBS to prepare a hydrogel with a concentration of 11% w/w. Drugs pre-dissolved in PBS had been added into the solution before it solidified. The final concentration of gel was 10% with a drug concentration of 0.5% of the total weight. The hydrogel was loaded into a syringe. 60 μg hydrogel was injected to the bottom of a 1.5 ml eppendorf tube. After that, 1 ml PBS was added to the tube. 500 μl of the solution was sampled at each time point and 500 μl fresh PBS was added. The concentration of the released DOXY was measured by quantifying the UV absorbance at 273 nm using UV-spectrophotometer (HITACHI, U-2910). The concentration was determined using a pre-established standard concentration-intensity curve. The concentration of released BSA was determined using a Micro BCA^{TM} Protein Assay Kit (Thermo Scientific) following the standard procedure.

Cell culture and cell viability in hydrogel. Articular cartilage was obtained from the femoral heads and knees (condyles and patellar grooves) of four-week-old New Zealand white rabbits (Harlan Sprague Dawley, Michigan, USA) under sterile conditions, stripped of any adherent connective tissue, and minced into small pieces. After digestion with 0.2% collagenase type II for 16 h, the primary chondrocytes were collected and were passaged for two times. The chondrocytes were cultured in a high glucose DMEM (Gibco) medium containing 20% (v/v) fetal bovine serum (FBS). Rabbit bone marrow-derived cells (BMSCs) were collected via aspiration from the femoral bone marrow using an 18-gauge syringe needle, collecting 10 ml of marrow into 1000 U of heparin. The marrow was filtered through a cell strainer to exclude fatty tissues and blood clots, and centrifuged at 600 rpm for 30 min. Rabbit BMSCs were collected and cultured in 75-cm^2 flasks in low-glucose α-MEM (Gibco) containing 10% fetal bovine serum (Gibco).

Before being dissolved in water, the DEX-UPy powders were sterilized by autoclaving at 121℃ for 25 min. Hydrogels (11%w/w) were prepared in PBS according to the above procedure. After the hydrogel solution was cooled down to room temperature, chondrocytes or BMSCs in a proper amount of medium were added and mixed while stirring, diluting the final concentration of the hydrogels to 10%. The cell density was 1 million/ml. The cell-hydrogel mixture was loaded into the syringe and injected into 12-well culture plate, followed by adding high glucose DMEM medium (Gibco) containing 20% fetal bovine serum (FBS). The culture medium was changed twice a week. Live/dead assay was conducted to evaluate the viability after two weeks of culture at 37℃ in an incubator with 5% CO_2. Before adding the assay solution, hydrogels

were washed with PBS for 3 times. One ml of live/dead assay solution (Invitrogen live/dead kit) was added to the culture plate and incubated at 37℃ in the incubator for 60 min. Samples were washed again with PBS and examined under confocal microscope (Olympus Fluoview 500).

Preparation of the self-integrated cell-gel constructs. Chondrocytes and BMSCs were encapsulated in two portions of the hydrogel separately with a final cell density of 1 million/ml. To better visualize the cells, chondrocytes and BMSCs were labeled with ER-Tracker™ Green (BODIPY® FL Glibenclamide, Invitrogen) and MitoTracker® Red CMXRos (Invitrogen), respectively, following the standard procedure. Then the cell-containing hydrogels were injected into the two sides of a disk-shaped PDMS mold (with the inner diameter of 4 mm, outer diameter of 7 mm, and thickness of 2 mm) separated by a baffle film (Teflon) in the middle. The film was subsequently removed to allow the integration of the chondrocyte-containing hydrogel and the BMSCs-containing hydrogel. To ensure sufficient time for complete gelation, the culture plate was put in the incubator for 60 min before adding the culture medium. The integrated hydrogel was observed under a confocal microscope (Olympus Fluoview 500) after being cultured in a DMEM medium (Gibco) with 20% FBS (without growth factors) for 2 days.

Subcutaneous implantation of cell-gel constructs. All animal procedures were carried out under the guidelines of the Institutional Animal Care and Use Committee of the University of Michigan. Nude mice (6–8 weeks old, NU/NU, Charles River Laboratories USA) were anaesthetized with 2.5% isoflurane in balanced oxygen. Three groups of cell-gel constructs (chondrocytes only, BMSCs/BMP-2 only, and self-integrated hydrogel with the two cell types on two sides) were fabricated using the same methods as described above. The cell density was 10

million/ml for both chondrocytes and BMSCs in all constructs. The concentration of BMP-2 was 50μg/ml. The cell-gel construct was implanted into subcutaneous pockets and each mouse received four implants. The implants were randomly arranged in nude mice, with four specimens per group. The constructs were collected after eight weeks and the fibrous capsules were removed. The samples were subsequently used for histological examinations.

Histologic examination of the implanted hydrogel constructs. The implanted specimens were collected and fixed in 10% buffered formalin at 4℃ for 8h. The fixed tissues were then immersed in Tissue-Tek™ CRYO-OCT compound (Sakura Finetek USA, Inc.) and subsequently stored at -80℃ overnight. The specimens were cryosectioned at a thickness of 10 μm and stained using Alcian blue and/or Alizarin red.

Reference:

[1] R. P. Sijbesma, F. H. Beijer, L. Brunsveld, B. J. B. Folmer, J. Hirschberg, R. F. M. Lange, J. K. L. Lowe, E. W. Meijer, Science 1997, 278, 1601.